

REGULATION OF THE CITRIC ACID CYCLE IN MAMMALIAN SYSTEMS

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1. Introduction

Whilst the reactions of the citric acid cycle [1] and related pathways are well established, the manner in which the flux through the pathway is regulated in different tissues remains an area of active research and debate. The primary focus during the last decade has been on enzyme mechanisms, the intracellular compartmentation of enzymes and intermediates of the cycle, and on the interrelations with the specific anion translocating systems of the inner mitochondrial membrane. A number of recent reviews have been concerned with relating citric acid cycle activity to the regulation of carbohydrate and fatty acid oxidation [2–4], metabolite transport across the mitochondrial membrane for gluconeogenesis, ureogenesis and fatty acid synthesis [5–11], cellular energy metabolism [12–16], as well as with more specialized topics such as pyruvate [17,18] and ethanol [19] metabolism. The concept that the enzymes of the citric acid cycle are organized as a functional multi-enzyme complex has also been advocated [20,21].

Until recently it has been generally accepted that turnover of the citric acid cycle per se is regulated at the dehydrogenase level primarily by the mitochondrial NAD^+/NADH ratio. This ratio is linked to the mitochondrial ATP/ADP ratio and ultimately to the cytosolic phosphorylation potential, which is a primary factor in the control of tissue respiration. However, since the different NAD^+ -substrate dehydrogenases are regulated with different sensitivities by the NAD^+/NADH ratio, the absolute NAD^+ redox potential for a given flux depends on the concentration and nature of the substrate supply to the mitochondria. On the other hand, the anaplerotic functions of the citric acid cycle are regulated by ancillary enzymes such as pyruvate dehydrogenase, pyruvate

carboxylase, carbamyl phosphate synthetase and glutamate dehydrogenase, which affect substrate delivery of cycle intermediates and ultimately the rate of production of end products such as glucose, fatty acids and urea. More recent observations have suggested that the activities of several citric acid cycle enzymes can be markedly influenced by low $[\text{Ca}^{2+}]$ [22]. This observation combined with the finding that the mitochondrial calcium content is hormonally regulated [23–25], has prompted the speculation that Ca^{2+} could also regulate the activity of the citric acid cycle. In this review, we provide a brief synopsis of feedback regulation in the citric acid cycle and examine the possibility of Ca^{2+} regulation in the context of current knowledge concerning the regulation of Ca^{2+} uptake and release by mitochondria and the likely range of intramitochondrial free $[\text{Ca}^{2+}]$.

2. Importance of regulation of the citric acid cycle

The citric acid cycle provides reducing equivalents to the electron-transport chain and also, via ancillary reactions, provides substrates for biosynthetic reactions in the cytosol. The relative importance of these roles in the overall metabolism of the tissue will thus vary with the particular organ and the dietary status of the animal. The basic reactions of the citric acid cycle and its interactions with ancillary functions such as gluconeogenesis and ureogenesis, which occur only in liver, are depicted schematically in fig.1 without reference to compartmentation of reactions between the cytosol and mitochondria. A particularly striking feature of the regulation of the citric acid cycle is its flexibility by which flux rapidly becomes adjusted to the metabolic demands of the tissue and the nature of the substrate mixture delivered to the organ. In heart and to a lesser extent in brain, the major role of the citric acid cycle is to accomplish oxidation of the acetyl moiety of acetyl-CoA to CO_2 and water, and

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

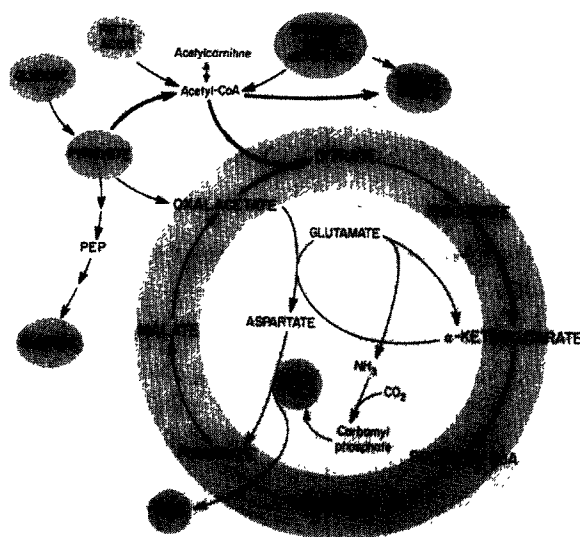


Fig.1. Schematic representation of the citric acid cycle showing interactions with major metabolic pathways in liver.

intermediates of the cycle are used catalytically such that, except for brief intervals during transition states, the individual enzymes behaves as a functional unit and cycle flux is proportional to oxygen consumption.

Cardiac muscle is able to oxidize a wide variety of substrates, including carbohydrate, fatty acids, ketone bodies and to a lesser extent amino acids [4]. Oxidation of these substrates, and particularly glucose, is subject to a variety of controls mediated by intracellular factors such as the ATP/ADP ratio, P_i , pH, $NAD^+/NADH$ ratio, as well as extracellular hormonal influences and the permeability characteristics of the plasma membrane. Consequently, the various substrates differ in their relative efficacy as respiratory fuels, which is determined ultimately by the maximum rate at which they can deliver acetyl-CoA to the citric acid cycle. Thus, when individual substrates are provided to the isolated perfused heart as sole exogenous respiratory fuel, and the heart is challenged by an increase of work load, respiration and hence the maximum work output reaches a limit, which varies with the nature of the substrate and is determined by the rate of acetyl-CoA generation [26]. Conversely, when acetyl-CoA utilization is below the saturation limit for different substrates, tissue acetyl-CoA and cycle intermediate levels vary over a wide range dependent on the substrate source with glucose as substrate, acetyl-CoA levels may be 10-fold lower than with fatty acid as substrate for the

same citric acid cycle flux [27–29]. Thus, it is clear that citric acid cycle activity is not regulated simply by the mitochondrial acetyl-CoA concentration.

The citric acid cycle, excluding pyruvate dehydrogenase which will be discussed later, contains three non-equilibrium enzyme steps, namely citrate synthase, NAD^+ -linked isocitrate dehydrogenase and α -ketoglutarate dehydrogenase. From studies with isolated mitochondria [30–34], together with metabolite analyses of hearts perfused under different conditions of substrate supply and work loads [27,29,35–38], an overall concept of regulation has been formulated (reviewed [4,6,15,16,39]). The key energy-linked regulatory factor determining citric acid cycle flux appears to be the mitochondrial free $NAD^+/NADH$ ratio, which interacts at a number of enzyme steps. The pyridine nucleotide redox state interacts with other intramitochondrial parameters such as the ATP/ADP, GTP/GDP, and $NADP^+/NADPH$, acetyl-CoA/CoASH, succinyl-CoA/CoASH, acetyl-CoA/succinyl-CoA and citrate/oxaloacetate ratios, in a manner which is dependent on the absolute concentrations of the cycle intermediates. Clearly the multiple and changing demands of tissue respiration and varied metabolic functions necessitate a precise, albeit complex, regulation of the individual enzymes of the citric acid cycle.

3. Coordination of cycle flux by feedback regulation

Citrate synthase activity is regulated directly by accessible pools of either of its two substrates oxaloacetate and acetyl-CoA. Oxaloacetate can be transported across the mitochondrial membrane by either the dicarboxylate [40] or α -ketoglutarate [41] translocator, but the unfavourable kinetic constants in conjunction with the low free oxaloacetate concentration in the mitochondria suggest that a direct communication between the cytosolic and mitochondrial pools of oxaloacetate is not an important factor in regulation of cycle activity. The apparent K_m of citrate synthase for oxaloacetate at saturating acetyl-CoA concentrations is 1–5 μM [20,42,43]. The mitochondrial oxaloacetate concentration is expected to be determined by the malate concentration and the $NAD^+/NADH$ ratio. Calculations of the oxaloacetate concentration of isolated heart mitochondria assuming equilibrium of malate dehydrogenase yielded 0.36 μM , while the value calculated from the mea-

sured concentrations of aspartate aminotransferase reactants was $10\ \mu\text{M}$ [34]. In these experiments, the measured mitochondrial oxaloacetate concentration for state 4 conditions was $36\ \mu\text{M}$. A similar problem arises from considerations of the oxaloacetate concentration in liver mitochondria [44]. It is generally accepted that mitochondrial oxaloacetate must be compartmented by protein binding, but it remains unresolved whether malate dehydrogenase and citrate synthase are both in equilibrium with the free mitochondrial oxaloacetate concentration, or whether either enzyme is displaced from equilibrium [4,6,16,34,45]. A concerted interaction between malate dehydrogenase and citrate synthase, which in effect results in substrate channeling, has been suggested [45]. Since citrate synthase in intact mitochondria or tissues operates well below its V_{max} , and flux is responsive to changes of the NAD^+/NADH ratio, it is concluded that the oxaloacetate concentration available to citrate synthase is in the regulatory K_m region [31,46–49]. Flux through citrate synthase in intact mitochondria is also effectively regulated by changes of acetyl-CoA concentration [50]. Kinetic studies with isolated citrate synthase show that the apparent K_m for acetyl-CoA is $5\text{--}10\ \mu\text{M}$ [20,42,43]. This is an order of magnitude lower than the range of acetyl-CoA concentrations found in isolated mitochondria [31], or calculated from total tissue contents assuming that most of the acetyl-CoA is mitochondrial [51]. The activity of citrate synthase is inhibited by physiological concentrations of NADH, NADPH and succinyl-CoA (competitive with acetyl-CoA and noncompetitive with oxaloacetate, [42,52]), which have the effect of increasing the apparent K_m for acetyl-CoA. Succinyl-CoA inhibition via the GTP/GDP ratio and succinate thiokinase provides an energy-dependent link between product inhibition of α -ketoglutarate dehydrogenase by succinyl-CoA and α -ketoglutarate generation through citrate synthase [6].

Regulation of acetyl-CoA entry into the citric acid cycle is however, not achieved solely through effects on citrate synthase activity, but rather by a concerted regulation of both citrate synthase and NAD^+ -linked isocitrate dehydrogenase, in an analogous manner to the coordinated regulation between hexokinase and phosphofructokinase mediated by the product inhibition of hexokinase by glucose 6-phosphate [53]. Thus, citrate synthase is inhibited by citrate competitively with oxaloacetate with $K_i\ 1.6\ \text{mM}$ which is well within the range of intramitochondrial citrate

concentrations [31]. The mitochondrial citrate concentration in turn is dependent on the activity of NAD^+ -linked isocitrate dehydrogenase, which is strongly regulated by the NAD^+/NADH ratio as a result of product inhibition by NADH [54,55]. The effect of this interaction is to allow an increase of flux despite a fall of the isocitrate concentration. During transitions from the resting to active state in isolated heart mitochondria (state 4–3 transition) or with an increased work load in perfused rat hearts, conditions associated with an oxidation of the mitochondrial pyridine nucleotides, a primary interaction at isocitrate dehydrogenase was identified by an immediate fall of citrate or isocitrate levels [30,31,35,56,57]. A fall of the citrate concentration results in a lowering of the apparent K_m of citrate synthase for oxaloacetate, which together with the direct effect on oxaloacetate levels as a consequence of the adjustment of the malate/oxaloacetate ratio by interaction of the increased NAD^+/NADH ratio with malate dehydrogenase, permits increased flux through citrate synthase.

In heart, an obligatory linkage of flux through citrate synthase to flux through isocitrate dehydrogenase is ensured by the low activity of the mitochondrial tricarboxylate carrier [30,47,58]. NAD^+ -linked isocitrate dehydrogenase is also allosterically activated by ADP in a Mg^{2+} -dependent manner [59], which together with an ATP inhibition of citrate synthase provided the basis for the concept of a direct adenine nucleotide regulation of citric acid cycle activity [50,60]. However, it is now fairly certain that changes of the mitochondrial ATP/ADP ratio are less important as a regulatory factor in mammalian mitochondria than changes of the NAD^+/NADH ratio for the following reasons:

- (i) As discussed in the following section, it is evident that over the physiologically relevant range of extramitochondrial ATP/ADP ratios associated with active mitochondrial respiration in the intact tissue, changes of the intramitochondrial ATP/ADP ratio are very small.
- (ii) The postulate of ATP inhibition of citrate synthase is no longer considered valid, primarily because MgATP , the predominant form of ATP in mitochondria, is not inhibitory [6,16].
- (iii) Studies with isolated heart mitochondria [30] and a soluble fraction from liver mitochondria [61] have indicated that regulation of flux through isocitrate dehydrogenase by the ATP/ADP

ratio is quantitatively much less than by the NAD^+/NADH ratio.

In contrast, the elegant studies by Hansford and colleagues (reviewed [16]) have shown very convincingly that regulation of the citric acid cycle in insect flight muscle is achieved primarily by ADP activation of isocitrate dehydrogenase rather than by changes of the NAD^+/NADH ratio, which in this specialized tissue falls rather than rises with increased respiratory activity.

Mitochondria also contain an NADP^+ -dependent isocitrate dehydrogenase, and various authors have discussed its participation in citric acid cycle turnover [4,10]. Increased flux through the NADP^+ -linked isocitrate dehydrogenase has been shown in isolated rat liver mitochondria after addition of uncoupling agents [62,63]. However, under energized conditions, the $\text{NADP}^+/\text{NADPH}$ ratio is maintained very low because of the high activity of the energy-linked transhydrogenase. Furthermore, the activity of NADP^+ regenerating reactions such as glutamate dehydrogenase is low in heart, so that flux through NADP^+ -linked isocitrate dehydrogenase probably contributes little to normal citric acid cycle flux in tissues with low biosynthetic functions. In liver, on the other hand, NADP^+ -linked isocitrate dehydrogenase has an important role:

- (i) It supplies NADPH for the reductive amination of ammonia and α -ketoglutarate to glutamate via glutamate dehydrogenase [64,65]; and
- (ii) It participates with cytosolic NADP^+ -linked isocitrate dehydrogenase in an isocitrate (citrate)— α -ketoglutarate cycle which equilibrates the cytosolic and mitochondrial NADP^+ redox potentials across the mitochondrial membrane [44,66].

This latter cycle can also be used to provide a net transport of NADPH reducing equivalents from mitochondria to the cytosol in response to a stimulation of cytosolic NADPH utilizing enzymes [67].

The mitochondrial NAD^+/NADH ratio interacts with the citric acid cycle at a third enzyme step, namely α -ketoglutarate dehydrogenase. As first noted by Garland [68], α -ketoglutarate dehydrogenase is inhibited by its products, NADH and succinyl-CoA. Further studies with purified enzyme from pig heart [33] showed that inhibition by succinyl-CoA was competitive with respect to CoASH (K_i 6.9 μM compared with a K_m for CoASH of 2.7 μM) and was independent of the NAD^+/NADH ratio. Inhibition by NADH was non-competitive with respect to both

NAD^+ and α -ketoglutarate, and was observed at both high and low succinyl-CoA levels. Independent or concurrent regulation of flux through α -ketoglutarate dehydrogenase by the succinyl-CoA/CoASH and NAD^+/NADH ratios has been described in experiments with both heart [30–32] and liver [33,69,70] mitochondria incubated under a variety of metabolic conditions (reviewed [6,16]). However, the inhibitory effects of high succinyl-CoA/CoASH or NADH/NAD^+ ratios can be overcome by an increase of the intramitochondrial α -ketoglutarate concentration, which appears to be in a favourable region for kinetic control of α -ketoglutarate dehydrogenase despite the fact that the apparent K_m for α -ketoglutarate observed with intact mitochondria is much higher than that observed for the isolated enzyme [32]. Recently a direct inhibitory effect of a high ATP/ADP ratio has been observed with purified α -ketoglutarate dehydrogenase [22,71] although whether this effect is important in isolated mitochondria remains to be ascertained. On the other hand, a relationship between the intramitochondrial ATP/ADP ratio and the succinyl-CoA/CoASH ratio (via the GTP/GDP ratio) has been observed in isolated liver and heart mitochondria [32,33]. This interaction, mediated through nucleotide diphosphate kinase and succinate thiokinase, allows the succinyl-CoA/CoASH ratio to be regulated independently of the NAD^+/NADH ratio, and may account for the fact that changes of the NAD^+/NADH ratio do not always correlate with changes of flux through α -ketoglutarate dehydrogenase [72].

Several points concerning regulation of α -ketoglutarate dehydrogenase are worth stressing since it not only is the enzyme important in the coordination of citric acid cycle activity, but α -ketoglutarate is a common intermediate among different pathways.

- (i) In transition states the activity of α -ketoglutarate dehydrogenase relative to that of isocitrate dehydrogenase is an important factor in permitting a redistribution of carbon between cycle intermediates in the mitochondria. The substrate concentrations of the individual enzymes thereby become adjusted to the new enzyme activity states with achievement of a common flux. Thus, increased flux is usually associated with decreased citrate and increased malate, and a consequent rise of the oxaloacetate concentration and ensuing substrate activation of citrate synthase [35].
- (ii) An additional feedback effect is exerted at citrate

synthase by the inhibitory effect of succinyl-CoA, which is most effective at high NAD^+/NADH ratios, low acetyl-CoA concentrations [31] and relatively high respiratory rates [30]. Further discussion of the role of the acetyl-CoA/succinyl-CoA ratio in the regulation of citric acid cycle activity is given in a review by Hansford [16].

- (iii) Since mitochondrial oxaloacetate and α -ketoglutarate are in equilibrium with the much larger pool sizes of aspartate and glutamate, differences of flux through citrate synthase and α -ketoglutarate dehydrogenase, in conjunction with transaminase activity, can lead to a net depletion or repletion of cycle intermediates [38]. A readjustment of enzyme activities in response to increased levels of acetyl-CoA from acetate [27] or NADH delivery from glycolysis [38] without appreciable changes of overall ATP generation in perfused rat heart is accompanied by increases in the total pool size of all cycle intermediates, and is associated with transient unequal flux through the span from acetyl-CoA to α -ketoglutarate compared with the span from α -ketoglutarate to oxaloacetate (reviewed [4,39]). Since the citric acid cycle per se does not permit net entry or removal of intermediates, this is achieved in skeletal or cardiac muscle by the combined action of alanine and aspartate aminotransferases by NAD^+ - and NADP^+ -linked malic enzymes, and by propionate and branched chain amino acid metabolism (e.g. [73,74]).

- (iv) During transfer of reducing equivalents from the cytosol to the mitochondria by the malate-aspartate cycle, α -ketoglutarate is formed in the mitochondrial matrix not only from isocitrate dehydrogenase but also as a product of transamination between oxaloacetate and glutamate.

There is thus a competition for α -ketoglutarate between further oxidation in the citric acid cycle and efflux to the cytosol in exchange with malate on the α -ketoglutarate carrier [39]. Steady-state fluxes through the malate-aspartate and citric acid cycles are achieved:

- (i) By adjustment of the mitochondrial α -ketoglutarate concentration, so that flux through α -ketoglutarate dehydrogenase, at its prevailing activity state determined by product inhibition becomes equal to that of citrate synthase and isocitrate dehydrogenase; and
- (ii) By adjustment of the cytosolic malate concentra-

tion (an activator) to meet the kinetic requirements of the malate: α -ketoglutarate translocator [39].

The experiments upon which these conclusions are based [38] have been relatively successfully modelled by computer simulation techniques [75].

4. Interrelations between the respiratory chain and the citric acid cycle

According to the generally accepted chemiosmotic coupling hypothesis of Mitchell [76,77], the respiratory carriers are arranged spatially in the inner mitochondrial membrane such that electron transport is associated with proton efflux from the mitochondrial matrix and the generation of a proton electrochemical gradient ($\Delta\tilde{\mu}_{\text{H}^+}$). This establishes an electrical potential, $\Delta\psi$ (negative inside), and a pH gradient (alkaline inside) across the inner mitochondrial membrane, with the overall protonmotive force (Δp) being defined by the relationship:

$$\Delta p = \Delta\tilde{\mu}_{\text{H}^+}/F = \Delta\psi - (2.3 RT/F) \Delta\text{pH}$$

Free energy is released in the oxidation-reduction reactions of the electron-transport chain by movement of reducing equivalents or electrons from a high electrochemical pressure (negative redox potential) to a lower electrochemical pressure (more positive redox potential). The proton electrochemical gradient provides a back pressure to the driving force produced by the oxidation-reduction reactions of electron transport, and thus regulates forward electron transport and the respiratory rate. Electron flow occurs when a proton circuit is established by inward proton transport. During ATP synthesis, inward movement of protons is channeled through the vectorial ATPase, which spans the mitochondrial inner membrane, and ATP is formed from ADP and P_i in the matrix space. ATP is subsequently transported out of the mitochondria in exchange for ADP on the adenine nucleotide translocator during ADP-stimulated respiration (state 3), and respiration slows to the controlled state 4 rate when the extramitochondrial adenine nucleotide phosphorylation potential achieves equilibrium with the protonmotive force. Energy invested in the maintenance of $\Delta\tilde{\mu}_{\text{H}^+}$ can also be used to support an electrophoretic influx of cations such as Ca^{2+} , or net efflux of negative charge associated with

anion transport, as in the electrogenic exchange of ADP^{3-} with ATP^{4-} . Energy is conserved during ion-transport, but respiration is not associated with ATP synthesis.

When mitochondria are incubated in the presence of oxidizable substrates, adenine nucleotides, phosphate and oxygen, the energetically useful redox potential difference from NADH to oxygen (ΔE_h) achieves thermodynamic equilibrium with a high cytosolic ATP phosphorylation potential, and electron transport flux is ideally zero. Because of the obligatory electrogenic nature of the adenine nucleotide translocator [78,79], the extramitochondrial ATP/ADP ratio is greater than that of the mitochondrial matrix according to the relationship:

$$\Delta\psi = \frac{2.3 RT}{F} \log \frac{(\text{ATP/ADP})_c}{(\text{ATP/ADP})_m}$$

With increased respiration, this equilibrium is disturbed by a fall of the cytosolic phosphorylation potential, with flux being proportional to the deviation from equilibrium between the driving force of the oxidation–reduction reactions and the extramitochondrial phosphorylation potential [13,15,80]. Since $(\text{ATP/ADP})_m$ falls proportionately much less than $(\text{ATP/ADP})_c$, the adenine nucleotide translocase deviates from equilibrium with $\Delta\psi$ during state 4–3 transitions with isolated mitochondria [81–83]. The concentration of free ADP in the cytosol is probably maintained within the operational K_m region for the adenine nucleotide translocator because of binding to actin and other proteins and by chelation with Mg^{2+} . An increased ADP concentration in the cytosol will thus stimulate ADP entry into mitochondria in exchange with ATP from the mitochondrial matrix in an attempt to re-establish the extramitochondrial ATP/ADP ratio [84], while the intramitochondrial ATP/ADP ratio is maintained relatively constant by the activity of the mitochondrial ATPase. Since the proton electrochemical gradient ($\Delta\tilde{\mu}_{\text{H}^+}$) provides a back pressure to the transport of electrons through the respiratory chain and is considered the energy-transducing intermediate between oxidation and phosphorylation, the fall of the intramitochondrial phosphorylation potential is accompanied by a fall of $\Delta\tilde{\mu}_{\text{H}^+}$. The near equilibrium relationship between ΔE_h and $\Delta\tilde{\mu}_{\text{H}^+}$ is thus disturbed and the rate of NADH oxidation by NADH

dehydrogenase is increased, which results in an oxidation of the NADH/NAD⁺ couple. As already discussed, the mitochondrial NAD⁺-linked substrate dehydrogenases are mostly irreversible enzymes that are kinetically regulated by the NADH/NAD⁺ ratio. Hence, each steady state increase of flux is associated with a new, more oxidized NAD⁺ redox potential as determined by the nature of the substrate supply and the sensitivity of the individual dehydrogenases to kinetic regulation by the NADH/NAD⁺ ratio. Increased electron transport causes a reduction of cytochrome *c* despite a contraction in the ΔE_h redox span from NADH to cytochrome *c* [85,86], which is important in the regulation of the cytochrome oxidase complex [87,88]. In summary, when respiration changes, the primary control signal is the cytosolic ATP/ADP ratio; this transmits its effects via changes of the transmembrane proton electrochemical potential and the mitochondrial NAD⁺/NADH ratio to the respiratory carriers and the substrate dehydrogenases, whose activity is thereby regulated secondarily to phosphate acceptor control of electron transport (further discussed in [15,16]).

5. Effects of Ca^{2+} on the activities of intramitochondrial enzymes

Considerable evidence suggests that Ca^{2+} plays a key role in controlling many biological functions and that mitochondria, which contain a high proportion of the total cellular calcium, may be crucially important in the control of intracellular Ca^{2+} homeostasis (reviewed [89,90]). Three mammalian mitochondrial dehydrogenases: the pyruvate dehydrogenase complex, NAD⁺-linked isocitrate dehydrogenase, and the α -ketoglutarate dehydrogenase complex, have been shown to be activated by Ca^{2+} [18,22,91], while Ca^{2+} has been shown to inhibit the activities of pyruvate carboxylase [92,93] and carbamyl-phosphate synthase [94].

The pyruvate dehydrogenase complex (PDH) catalyses the irreversible oxidation of pyruvate to acetyl-CoA according to the reaction:



In heart muscle, essentially all the acetyl-CoA formed from pyruvate is oxidized via the citric acid cycle whereas in other tissues such as liver and adipose tis-

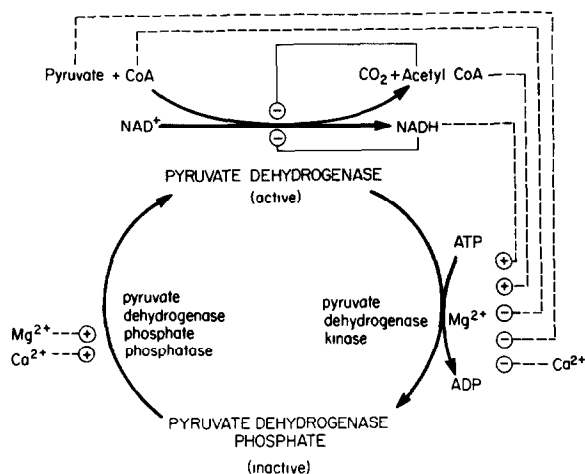


Fig.2. Mechanisms of regulation of pyruvate dehydrogenase by end-product inhibition and by phosphorylation-dephosphorylation, and their interrelation.

sue, a substantial proportion of the acetyl-CoA is utilized for the biosynthesis of fatty acids and sterols. Pyruvate dehydrogenase is thus involved in both bioenergetic and biosynthetic pathways and it is therefore not surprising that the complex is subject to precise control by end-product inhibition and by a phosphorylation-dephosphorylation cycle, with interrelations between the two types of control (reviewed [4,18,95]). Fig.2 summarizes the regulatory properties of PDH; the reaction is inhibited (through end-product inhibition) by high ratios of acetyl-CoA/CoASH and NADH/NAD⁺. In general, the principal substrates for the PDH reaction are also inhibitors of the kinase reaction and the product inhibitors of the dehydrogenase reaction are also activators of the kinase reaction. Free Ca²⁺ has been shown to affect the interconversion of the active and inactive forms of pyruvate dehydrogenase; PDH phosphatase from a number of tissues requires Mg²⁺ and is activated by Ca²⁺ with K_m about 0.7 μ M [91], while the kinase is inhibited at $>0.1 \mu$ M Ca²⁺ [96]. However, a substantially higher K_m of Ca²⁺ for the phosphatase reaction has also been reported [97]. Most experiments studying the Ca²⁺ sensitivity of PDH have utilized Ca-EGTA buffers and relied on computed free Ca²⁺ concentrations. In the limited number of experiments where Ca²⁺-dependence was demonstrated without using Ca-EGTA buffers, substantially higher concentrations of added Ca²⁺ were required to elicit effects on PDH kinase and phosphatase activities [91,96].

The reason for this discrepancy has not been resolved, but might be related to artifacts induced by the presence of EGTA through chelation of other ionic species. Studies with intact heart, liver and adipose tissue mitochondria have generally confirmed an activation of PDH by Ca²⁺. Depletion of calcium from isolated mitochondria by addition of the Ca²⁺-ionophore, A-23187, led to inactivation of PDH phosphatase, which could be reversed by the addition of both Mg²⁺ and Ca²⁺ [98,99]. Upon varying the extramitochondrial Ca²⁺ by the use of Ca-EGTA buffers the [Ca²⁺] required for half-maximal effect on PDH was found to be $<1 \mu$ M [100,101].

The proportion of active (non-phosphorylated) pyruvate dehydrogenase is decreased by starvation and by alloxan-diabetes in heart, skeletal muscle, liver, kidney and adipose tissue [102–106] with no change in the total activity. This effect could not be explained by changes of the acetyl-CoA/CoASH or NAD⁺/NADH ratios. Studies with mitochondria or extracts from alloxan-diabetic or starved animals showed that the kinase activity was insensitive to inhibition by pyruvate and that there was an apparent inhibition of phosphatase activity due to a change in K_m of the PDH substrate for the phosphatase [104, 107,108]. It has been suggested that in the rat, diabetes and starvation are associated with multi-site phosphorylation of the decarboxylase tetramer ($\alpha_2\text{P}_3\beta_2$) of PDH [107,108]. In addition, the effect of phosphorylation at sites 2 and 3 on phosphatase is to cause about a 3-fold increase in the apparent K_m for Ca²⁺, suggesting a possible link with Ca²⁺ in these metabolic states [97].

The activity of NAD⁺-linked isocitrate dehydrogenase has recently been shown to be activated by Ca²⁺ with a half-maximal effect at about 1 μ M [109]. The reported inhibition by Ca²⁺ of the enzyme from rat heart [110] has been attributed to the removal of Mn²⁺, which is obligatory for enzyme activity, by the EGTA in the Ca-EGTA buffer [109]. The effects of Ca²⁺ on NAD⁺-linked isocitrate dehydrogenase are only observed in the presence of ADP [109]; it is possible that the effect of Ca²⁺ may be related to the reported association of the enzymically active 330 000 mol. wt protomer in the presence of ADP to dimeric and tetrameric forms [111,112]. No effects of Ca²⁺ were observed on the activity of mitochondrial NADP⁺-linked isocitrate dehydrogenase [109]. In an attempt to demonstrate the Ca²⁺-sensitivity of NAD⁺-linked isocitrate dehydrogenase with intact mitochondria,

McCormack and Denton [113] examined the effects of changes in extramitochondrial Ca^{2+} (using Ca-EGTA buffers) on the oxidation of *threo*-D₃-isocitrate in uncoupled mitochondria from brown adipose tissue. The half maximal concentration required for Ca^{2+} activation was 1.6 μM , and Ca^{2+} lowered the apparent K_m for isocitrate about 3-fold. Nevertheless, the elevated concentrations of mitochondrial ATP in the uncoupled state are such as to favour the Ca^{2+} -dependence of the enzyme.

α -Ketoglutarate dehydrogenase from heart and kidney is also activated by Ca^{2+} in the 1 μM range based on Ca-EGTA buffers; in the absence of NADH, Ca^{2+} caused a decrease of the apparent K_m for α -ketoglutarate [22,71]. In the presence of NADH, Ca^{2+} decreased the NADH inhibition of α -ketoglutarate dehydrogenase, and produced a 20-fold increase in the activity of α -ketoglutarate dehydrogenase over 10^{-7} – 10^{-5} M Ca^{2+} , whereas in the absence of NADH there was only a 2-fold increase [71]. The apparent K_m for α -ketoglutarate is increased by ATP and decreased by ADP [22,71]. These effects appear to be independent of the Ca^{2+} effect on the apparent K_m for α -ketoglutarate. However, in experiments where the ATP/ADP ratio was altered, the effect of Ca^{2+} on the NADH inhibition was considerably diminished; as the ATP/ADP ratio decreased, the NADH inhibition became less sensitive to the presence or absence of Ca^{2+} [71]. It is of interest to note that although increased Ca^{2+} promotes flux through both pyruvate and α -ketoglutarate dehydrogenases, this effect is achieved by different mechanisms. In the case of pyruvate dehydrogenase the activation by Ca^{2+} appears to be mediated by lowering the K_m of the phosphorylated enzyme for the phosphatase and also by inhibiting the kinase, thereby increasing the proportion of enzyme in the active form. In contrast the effect of Ca^{2+} on α -ketoglutarate dehydrogenase appears to be mediated by a decrease in the apparent K_m for α -ketoglutarate and, perhaps more significantly by decreasing the NADH inhibition of the enzyme. Certainly the effects of Ca^{2+} activation, mediated through effects on interconverting enzymes (kinase/phosphatase of pyruvate dehydrogenase), or binding of effectors (ADP to isocitrate dehydrogenase; NADH to α -ketoglutarate dehydrogenase) offers an interesting possibility for effecting a concerted regulation of flux through the citric acid cycle. As discussed in the following sections, the critical factor is whether the mitochondrial free Ca^{2+} is normally in

the correct range for regulation, and whether it changes under different metabolic conditions in a manner to account for alterations of citric acid cycle activity.

By comparison with the dehydrogenases, the effects of Ca^{2+} on carbamyl phosphate synthetase and pyruvate carboxylase are less well documented. In rat liver mitochondria, citrulline synthesis is stimulated by EGTA and strongly inhibited by addition of low $[\text{Ca}^{2+}]$ [94]. Further studies showed that the inhibition of carbamyl-phosphate synthetase by Ca^{2+} occurred over the range of physiological mitochondrial calcium contents and could be counteracted by increased Mg^{2+} (A. J. Meijer and J. R. Williamson, unpublished). However, the mechanism of the Ca^{2+} inhibition remains to be established; it appears to be independent of changes of *N*-acetylglutamate, an obligatory activator of carbamyl-phosphate synthetase. In many respects the regulation of carbamyl-phosphate synthetase resembles that of pyruvate carboxylase; both enzymes require specific allosteric activators (*N*-acetyl-glutamate and acetyl-CoA, respectively), MgATP and free Mg^{2+} for activation and are inhibited by Ca^{2+} , competitive with Mg^{2+} . Recent studies with isolated liver mitochondria have indicated that low concentrations of Ca^{2+} inhibited pyruvate carboxylation under conditions where there was no change in the intramitochondrial ATP, ADP or acetyl-CoA levels but a concomitant increase in the proportion of active pyruvate dehydrogenase [114]. Depletion of mitochondrial Ca^{2+} would thus be expected to stimulate both urea synthesis and gluconeogenesis.

6. Regulation of intramitochondrial Ca^{2+} homeostasis

Cells generally contain 1–2 μmol total calcium/g wet wt, most of which is sequestered in the intracellular organelles. Rapid disruption techniques applied to isolated hepatocytes have shown that 60–80% of the total calcium is in the mitochondria with most of the remainder being associated with the endoplasmic reticulum [25]. The cytosolic free Ca^{2+} of cells in the unstimulated state has generally been assumed to be 10^{-7} – 10^{-6} M, largely on the basis of the known threshold values for stimulation of Ca^{2+} -sensitive enzymes, Ca^{2+} -binding proteins or Ca^{2+} -sensitive metabolic or physiological functions of cells with a disrupted plasma membrane [115–117]. Recent direct mea-

measurements of cytosolic free Ca^{2+} in squid axon [118], muscle [118–121] and isolated hepatocytes [25] have provided values of 40–200 nM under control conditions and have indicated that the increase following stimulation is relatively modest (<5-fold).

The mechanisms of Ca^{2+} transport by isolated mitochondria have recently been defined (reviewed [90,123]). Uptake and efflux of Ca^{2+} occurs by two separate and independent systems. Calcium uptake occurs by the electrophoretic transport of Ca^{2+} down the electrochemical gradient generated by the electrogenic efflux of protons produced by flux through the electron-transport chain, as illustrated in fig.3. The translocator has a high V_{max} and K_m 2–20 μM , depending on the mitochondrial species; the presence of Mg^{2+} increases the apparent K_m . Calcium efflux occurs by an electroneutral exchange of Ca^{2+} with H^+ or Na^+ [90,124–126]. The V_{max} for Ca^{2+} efflux is much lower than that for Ca^{2+} influx, while the

apparent K_m is higher [127]. Isolated mitochondria have been shown to buffer the external free Ca^{2+} concentration, which remains approximately constant under defined conditions of Mg^{2+} , Na^+ and energy state as a result of net uptake or release of mitochondrial calcium until influx and efflux rates become equal. At equilibrium, a free $[\text{Ca}^{2+}]$ of 0.8 μM has been observed with liver mitochondria, which was increased by addition of 1 mM Mg^{2+} [128], while with heart mitochondria, 0.2 μM and 1.4 μM were observed in the absence and presence of 5 mM Na^+ [125]. The simultaneous operation of independent uptake and efflux pathways across the inner membrane results in a continuous cycling of Ca^{2+} between the cytosolic and matrix components, which is thus associated with a continuous, albeit small, dissipation of $\Delta\tilde{\mu}_{\text{H}^+}$.

On a thermodynamic basis, according to the Nernst equation:

$$\Delta\psi = \frac{2.3 RT}{2 F} \log \frac{[\text{Ca}^{2+}]_{\text{m}}}{[\text{Ca}^{2+}]_{\text{c}}}$$

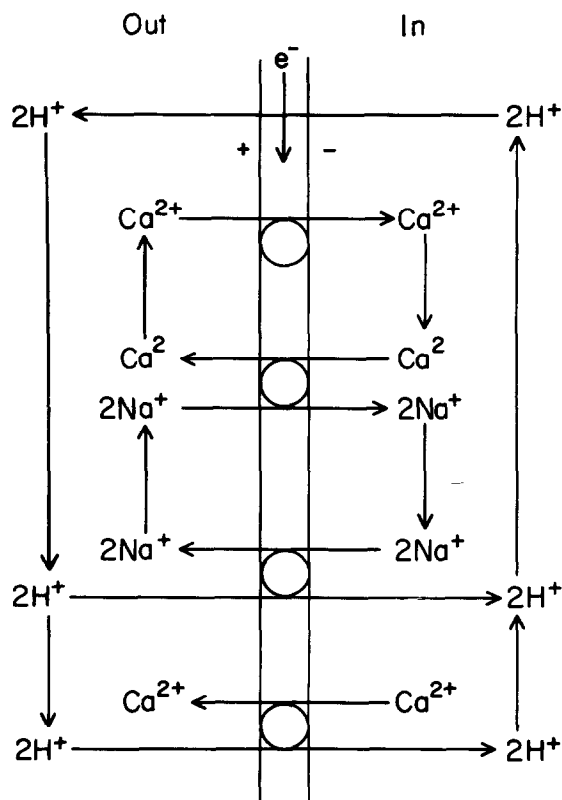


Fig.3. Schematic representation of mitochondrial Ca^{2+} -transport involving: (a) electrophoretic influx down the proton electrochemical gradient; and (b) Ca^{2+} -efflux by electroneutral exchange with H^+ or Na^+ .

a 10^5 gradient of Ca^{2+} across the inner mitochondrial membrane would be predicted at equilibrium for $\Delta\psi$ 150 mV. However, it is evident that Ca^{2+} gradients of this magnitude are not attained by isolated mitochondria. A study of the relationship between extramitochondrial free Ca^{2+} and Ca^{2+} accumulated in the matrix indicated a biphasic relationship; the extramitochondrial Ca^{2+} was almost independent of the Ca^{2+} accumulated in the matrix up to about 50 nmol Ca^{2+} /mg protein but further accumulation was associated with a rise in the extracellular Ca^{2+} [128]. Direct measurements of the mitochondrial free $[\text{Ca}^{2+}]$ have yielded $64 \pm 2 \mu\text{M}$ for liver mitochondria containing 10 nmol total calcium/mg protein [127]. Measurement of the total mitochondrial Ca^{2+} following rapid disruption of isolated hepatocytes provided a similar value of 9.8 nmol calcium/mg mitochondrial protein [25]. Furthermore, studies on the relationship between the total calcium content of mitochondria and the free $[\text{Ca}^{2+}]$ achieved by calcium loading of the mitochondria, indicated a linear relationship between free Ca^{2+} versus total calcium over 10–36 nmol/mg mitochondrial protein with a slope of 0.004 [127]. Mitochondrial free Ca^{2+} at 64 μM and cytosolic free Ca^{2+} at 0.2 μM [25] implies a gradient of free Ca^{2+} across the mitochondrial membrane in the hepatocyte of 320, which corre-

sponds to a $\Delta\psi$ of 75 mV according to the Nernst equation. This value may be compared with about 80 mV for $\Delta\psi$ obtained by Nicholls [128] below which liver mitochondria were unable to maintain extramitochondrial steady state free Ca^{2+} at 0.8 μM , as $\Delta\psi$ was decreased by increasing the $[\text{K}^+]$ in the presence of valinomycin. These and earlier data [129,130] suggest that the gradient of free Ca^{2+} across the mitochondrial membrane is in equilibrium with the membrane potential up to 70–80 mV and thereafter becomes independent. The agreement between the numbers generally supports the validity of the directly measured Ca^{2+} gradient. However, the measurement of the matrix free $[\text{Ca}^{2+}]$ relies on a determination of the medium free $[\text{Ca}^{2+}]$ at the null point, when Ca^{2+} movements across the mitochondrial membrane are zero in the presence of ionophore A-23187, and assumes that there is no $[\text{Ca}^{2+}]$ gradient under these conditions. This assumption is supported by the lack of effect of nigericin (added to promote collapse of the ΔpH) on the measured Ca^{2+} null point values, and a negligible ΔpH calculated from the distribution of $[\text{C}^{14}]\text{DMO}$ (5,5'-dimethyl-oxazolidine-2,4-dione). Nonetheless, a residual ΔpH of 0.15 units would correspond to a 2-fold Ca^{2+} gradient, but it is unlikely that corrected matrix free Ca^{2+} would be $<30 \mu\text{M}$.

7. Physiological significance of intramitochondrial Ca^{2+} in relation to activity of the citric acid cycle

In attempting to assess whether intramitochondrial Ca^{2+} is likely to play a role in mediating flux through the citric acid cycle, the intramitochondrial $[\text{Ca}^{2+}]$ relative to the Ca^{2+} -sensitivity of the particular enzymes is clearly of paramount importance. Matrix free Ca^{2+} at about 60 μM obtained with isolated liver mitochondria [25,127] is considerably higher than the reported Ca^{2+} -sensitivity range of isolated NAD⁺-linked isocitrate dehydrogenase, α -ketoglutarate dehydrogenase and pyruvate dehydrogenase, although in the latter case there is evidence to suggest that under certain circumstances the Ca^{2+} sensitivity might be in a range favourable for kinetic control through changes in intramitochondrial free Ca^{2+} . Certainly in the case of NAD⁺-linked isocitrate dehydrogenase and α -ketoglutarate dehydrogenase, further studies are warranted to establish fully the range of Ca^{2+} over which the enzymes respond; the studies with Ca-EGTA

buffers certainly do not rule out the possibility that EGTA itself may affect the Ca^{2+} -sensitivity. The studies on the effect of changes in extramitochondrial Ca^{2+} on the activities of pyruvate dehydrogenase, NAD⁺-linked isocitrate dehydrogenase and α -ketoglutarate dehydrogenase in uncoupled mitochondria [101,113] also do not provide reliable indications of changes in intramitochondrial Ca^{2+} to which the enzymes may be responding, since it is possible that under the conditions of the experiments a gradient of free Ca^{2+} across the inner mitochondrial membrane determined by a residual membrane potential ($\Delta\psi$) could still exist; a contribution of 30 mV could lead to a 10-fold gradient.

It is pertinent to consider whether changes in intramitochondrial Ca^{2+} can be correlated with changes in citric acid cycle flux. Unfortunately, the available data are contradictory. The best documented hormone-induced effects on hepatic calcium homeostasis are those mediated by α -adrenergic agents and vasopressin, which cause a fall of mitochondrial total [23–25] and free calcium (J. R. Williamson, unpublished). This effect might account for the observed stimulation of pyruvate carboxylation with phenylephrine [131] as a result of a diminished inhibition of pyruvate carboxylase by Ca^{2+} , but is inconsistent with a Ca^{2+} -mediated regulation of citric acid cycle activity, which increases despite a fall of mitochondrial Ca^{2+} . Furthermore, vasopressin effects on liver include an activation of pyruvate dehydrogenase [132], rather than an inhibition predicted from the lowered mitochondrial Ca^{2+} . On the other hand, glucagon stimulates gluconeogenesis and citric acid cycle activity, but apparently has no effect on cytosolic free Ca^{2+} [25] or mitochondrial Ca^{2+} [125]. Whether the increased activity of pyruvate dehydrogenase and citric acid cycle flux associated with increased work load in heart and gastrocnemius muscle [103,133,134] is in any way associated with changes in mitochondrial Ca^{2+} levels remains, for the moment, purely speculative. It has been reported that increased oxidation of intramitochondrial pyridine nucleotides induces a net efflux of Ca^{2+} from liver mitochondria [135,136]; it is therefore possible that the oxidation of pyridine nucleotides associated with conditions of increased work load may actually lead to a lowering of mitochondrial free Ca^{2+} . Certainly before definitive statements can be made as to whether Ca^{2+} may provide another locus of control for the citric acid cycle, interrelated with the well-

documented feedback regulation by NAD^+/NADH , a more comprehensive study of the intramitochondrial Ca^{2+} -levels, under a variety of metabolic and hormonal states, is required. The limited evidence available at present pointing to a disparity between intramitochondrial Ca^{2+} -levels and the Ca^{2+} -sensitivity of enzymes of the citric acid cycle would tend to argue against such a regulatory role, although regulation of pyruvate dehydrogenase, pyruvate carboxylase and carbamyl phosphate synthetase activities by Ca^{2+} remains a distinct possibility. Our present conclusion on the basis of available evidence is that the mitochondrial free Ca^{2+} is likely to be always in the range to saturate isocitrate and α -ketoglutarate dehydrogenases, and that Ca^{2+} binding serves the purpose of modulating the inhibitory effects of high NADH/NAD^+ and ATP/ADP ratios.

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